

### Method of Peptide Synthesis

The chemical synthesis of peptides up to 40 residues is now routinely efficient and recent advances over the last 10 years has led to the synthesis of peptides and small proteins in the range of 40-150 residues. Efficient novel synthetic methodology and a wide array of resins which can be used for synthesis have contributed to this.

One particular resin, developed by Wang, S.S. *J.Amer.Chem.Soc.* **95**, (1973), 1328, (see figure 1) has become the industry standard which has proven effective in the efficient synthesis of long peptides. There are however a number of problems with this resin which relate to the C-terminal amino acid. Firstly esterification of the resin with protected derivatives of cysteine and histidine can cause significant levels of racemisation which, of course, is highly undesirable. Further, whilst esterification with

1 protected derivatives of proline is successful  
2 problems are encountered after an additional amino  
3 acid residue is added to form a dipeptide.  
4 Deprotection of the dipeptide in preparation for  
5 the coupling of the third amino acid gives a free  
6 amino dipeptide ester which often cyclises  
7 internally to form the free cyclic dipeptide (a  
8 diketopiperazine) shown in figure 2. The resultant  
9 loss of dipeptide is in most cases quantitative and  
10 renders use of the Wang resin unsuitable for the  
11 synthesis of C-terminal proline peptides. Moreover  
12 it has also been suggested that cyclisation also  
13 occurs when the penultimate C-terminal residue is a  
14 proline residue or one of its derivatives.

15  
16 The use of the sterically hindered and extremely  
17 acid labile 2-chlorotrityl chloride resin (see  
18 figure 3) is recommended for the synthesis of C-  
19 terminal proline containing peptides (as the steric  
20 bulk inhibits diketopiperazine formation).

21  
22 Experiments were carried out to synthesise medium  
23 length and long peptides where, due to the nature  
24 of the C-terminal residue, 2-chlorotrityl resin was  
25 used. The medium length peptide (about 30 residues)  
26 was HNP-1 where the C-terminal residue is cysteine  
27 The long peptide was guinea pig eotaxin, a 74 amino  
28 acid peptide, of which the C-terminal residue is  
29 proline.

30  
31 Both experiments were unsuccessful. Low yields of  
32 both peptides were obtained and monitoring of the

1 chain assembly showed a low coupling efficiency in  
2 both cases. By comparison with the situation when  
3 the HNP-1 peptide was synthesised on a Wang resin  
4 using a resin loading procedure that was reported  
5 to alleviate the problem of racemisation of C-  
6 terminal cysteine, the chain assembly proved  
7 excellent and the low yield obtained with the  
8 chlorotrityl resin was ascribed to some property of  
9 that resin.

10

11 One theory was that the extreme acid lability of  
12 this resin led to a premature cleavage of the  
13 peptide from the resin during chain assembly. The  
14 inventors varied the conditions of synthesis to try  
15 to eliminate the contact of the resin with acid  
16 species during chain assembly of guinea pig eotaxin  
17 but no improvement in yield was achieved. Another  
18 theory is that some property of the 2-chlorotrityl  
19 resin, e.g. swelling characteristics, renders it  
20 unsuitable and inefficient in the assembly of long  
21 peptides.

22

23 Thus 2-chlorotrityl resin appears only compatible  
24 with the synthesis of relatively short ( e.g. <20  
25 residues) peptides. It has now been found that the  
26 problems associated with respect to a peptide  
27 containing a C-terminal proline on 2-chlorotrityl  
28 resin can be alleviated if the synthesis is carried  
29 out on the Wang resin.

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**1 Summary of the Invention**

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3 The invention relates to a method for synthesis of  
4 a given peptide which contains a proline or one of  
5 its derivatives, at proximity to, or at, the C-  
6 terminus end of the peptide of interest. This  
7 method is particularly suitable for the synthesis  
8 of long peptides, for example peptides which have  
9 at least 20 amino acid residues or for peptides  
10 where synthesis is problematic on 2-  
11 chlorotritylchloride resin.

12

13 By the expression "proximity to" it is meant that  
14 the proline residue is positioned at the  
15 penultimate C-terminal position.

16

17 The expression "derivatives" is directed to a  
18 peptide, an amino acid or an amino acid residue  
19 which may differ from the corresponding peptide  
20 amino acid or residue by the substitution/addition  
21 of various substituents. It is usual in protein  
22 synthesis to use modified amino acids having  
23 protecting groups or which have been modified so as  
24 to be able to act as labels or tags or for other  
25 desirable purposes. For example, in the method of  
26 the present invention amino acid derivatives such  
27 as hydroxyproline or other proline derivatives  
28 could be used.

29

30 In a preferred embodiment, the method comprises the  
31 steps of:

- 1           a)   synthesising on a first resin a C-  
2               terminal portion of said peptide, or its  
3               derivative, comprising at least three  
4               successive amino acid residues or their  
5               derivatives, by successive coupling of  
6               selected amino acids, small peptides or  
7               their derivatives, said first resin being  
8               suitable for the formation of peptides  
9               having a proline residue or a proline  
10              derivative positioned at, or at proximity  
11              of, the C-terminal end of said peptide;  
12           b)   cleaving the C-terminal portion thus  
13               obtained from said first resin;  
14           c)   reattaching said C-terminal portion to a  
15               second resin which is generally suitable  
16               for the synthesis of peptides but is  
17               unsuitable for the formation of peptides  
18               having a proline residue or a proline  
19               derivative positioned at, or at proximity  
20               of, the C-terminal end of said peptide;  
21               and  
22           d)   coupling selected amino acids, small  
23               peptides or derivatives to the C-terminal  
24               portion to obtain said given peptide.

25  
26   Whilst peptides of any length can be synthesised  
27   using the method of the invention, the method is  
28   particularly suited for the synthesis of peptides  
29   having at least 20 amino acid residues or "long  
30   peptides". The method is particularly suitable for  
31   peptides having up to about 150 amino acid  
32   residues.

1 The method of the invention allows synthesis of  
2 peptides which were otherwise difficult to obtain  
3 quantitatively. Amongst such peptides which have a  
4 C-terminal proline residue and can be obtained  
5 using the method of the invention chemokines are of  
6 particular interest and particularly the human  
7 chemokines IP-10, BLC and MCP-2.

8  
9 Advantageously, the first resin is chosen so that  
10 it does not lead to the formation of cyclic  
11 dipeptides and in particular to the formation of  
12 diketopiperazine compounds.

13  
14 Step a) and/or d) of the method of the invention  
15 may be achieved by successive coupling of the  
16 predetermined amino acid residues, small peptides  
17 or their derivatives. This can be carried out  
18 using standard solid phase procedures which are  
19 well known. In these procedures, the  $\alpha$ -amino group  
20 of the next selected amino acid or small peptide is  
21 protected using a protecting group and is added to  
22 the resin bearing the C-terminal portion of the  
23 peptide together with a coupling agent like  
24 diisopropylcarbodiimide (DIC) or  
25 dicyclohexylcarbodiimide (DCC). The  $\alpha$ -amino  
26 protecting group is then removed by exposure to a  
27 suitable base which leaves the peptide bond intact  
28 and the next amino residue can then be added by  
29 repeating the above step. Such procedures are  
30 detailed for example in W.C. Chan and P.D. White,  
31 Fmoc Solid Phase Peptide Synthesis A Practical  
32 Approach, OUP 2000.

1 A preferred first resin for the formation of the C-  
2 terminal portion is the 2-chlorotrityl chloride  
3 resin or any similar resin which inhibits or  
4 minimises the formation of diketopiperazine.

5

6 A preferred resin to be used as the second resin  
7 for synthesis of a long peptide which can be used  
8 in the method of the invention is a resin having  
9 benzyl ester linker like the 4-(3-methoxy-4-  
10 (hydroxymethyl)phenoxy-methyl) derivative of  
11 polystyrene-co-divinylbenzene which is marketed  
12 under the Trade Mark SASRIN™. A particularly  
13 preferred resin is a 4-Hydroxymethylphenoxy-methyl  
14 resin known as Wang resin. Wang resins are well  
15 known and widely available.

16

17 Advantageously, the cleaving step from the first  
18 resin is achieved using a mild acid treatment, for  
19 example 20% trifluoroethanol in dichloromethane.  
20 This allows a fully protected (tri-) peptide moiety  
21 to be obtained. Thus, the C-terminal portion can  
22 be provided fully protected so it can be coupled  
23 directly onto the resin suitable for synthesis of a  
24 long peptide. The protective groups may be the  
25 standard protective groups usually used in Fmoc (9-  
26 fluorenylmethoxycarbonyl), Nsc (2-(4-  
27 nitrophenylsulfonyl)ethoxycarbonyl) or t-Boc (ter-  
28 butyloxycarbonyl) peptide synthesis.

29

30 The invention will now be described by way of  
31 example only, with respect to figures in which:

32

1 Figure 1: shows molecular structure of the Wang  
2 resin linker.

3 Figure 2: shows formation of diketopiperazine.  
4

5 Figure 3: shows molecular structure of the 2-  
6 chlorotrityl chloride resin linker.  
7

#### 8 **Example**

9  
10 The synthesis of guinea pig eotaxin, which contains  
11 a C-terminal proline residue, has been achieved  
12 using this resin exchange technique with an overall  
13 yield of 5mg following purification and disulphide  
14 bond formation. When one considers that the same  
15 scale synthesis performed on a 2-chlorotrityl resin  
16 typically yields < 1mg overall, the advantages of  
17 the method according to the invention are clearly  
18 evident.  
19

20 Any protein/peptide susceptible to diketopiperazine  
21 formation can be assembled using this described  
22 strategy. Polypeptides or proteins that contain  
23 proline or proline derivatives at, or adjacent to,  
24 the C-terminus are susceptible to diketopiperazine  
25 formation during assembly. The described approach  
26 will be extremely enabling for the synthesis of  
27 such peptides.  
28

29 Synthesis of gp eotaxin protected C-terminal  
30 tripeptide on 2-chlorotrityl resin (Fmoc-Thr(Bu<sup>t</sup>)-  
31 Lys(Boc)-Pro-ClTrtR) (1)  
32

1 Peptide synthesis was carried out on the ABI 430A  
2 peptide synthesiser. H-Pro-2-chlorotrityl resin  
3 (1g, 0.49mmol/g, Lot no. PrT-2, Nankai Hecheng Co.  
4 Ltd., China) was used in the reaction vessel. Nsc-  
5 Lys(Boc)-OH (503mg, 1mmol) was activated with HOCT  
6 (4ml, 1mmol, GL Biochem, (Shanghai) Ltd. China) and  
7 DIC (4ml, 1mmol, Acros) for 15mins then transferred  
8 to the reaction vessel and coupled for 30mins. A  
9 second cartridge of Nsc-Lys(Boc)-OH was activated  
10 similarly and recoupled to the resin after draining  
11 the first solution.

12

13 Following capping of unreacted amino groups on the  
14 resin with acetic acid anhydride (0.5M in DMF,  
15 10ml) the Nsc group was removed with Deblock  
16 solution (1% DBU, 20% piperidine in DMF).

17

18 Fmoc-Thr(But)-OH (397mg, 1mmol, Applied Biosystems)  
19 was activated in the same manner and coupled to the  
20 resin for 30mins followed by recoupling of the same  
21 amino acid as before. After coupling the resin was  
22 washed with DMF then DCM and dried under vacuum  
23 giving a yield of 1.21g of (1).

24

25 The synthesis was repeated using a further gram of  
26 resin furnishing 1.18g of the title resin. The  
27 resin batches were combined for further work.

28

29 Cleavage and isolation of Fmoc-Thr(Bu<sup>t</sup>)-Lys(Boc)-  
30 Pro-OH (2)

31

1 The peptide resin (1) was stirred in a solution of  
2 trifluoroethanol (20%) in DCM (50ml) for 60mins.  
3 The resin turned dark green. The solution was  
4 filtered and evaporated under reduced pressure to  
5 give an oil which was triturated with cold diethyl  
6 ether / hexane. The solvent was evaporated and  
7 fresh hexane added to yield a solid from which the  
8 solvent was again removed by evaporation. A white  
9 solid (400mg, 0.55mmol) was obtained. Mass  
10 spectroscopy Electrospray positive ion found 723.4,  
11 expected for  $C_{39}H_{54}N_4O_9$  722.4 kD.

12

13 Coupling of (2) to Wang resin to give Fmoc-  
14 Thr(Bu<sup>t</sup>)-Lys(Boc)-Pro-O-Wang resin (3)

15

16 The protected tripeptide (2) (400mg, 0.55mmol) was  
17 dissolved in the minimum volume of DMF (<2ml) and  
18 activated by the addition of DIC (86 $\mu$ l, 0.55mmol)  
19 and sonicated for 15mins.

20

21 Wang resin (800mg, 0.56mmol/g, Lot no. W-34,  
22 Nankai Hecheng Co. Ltd., China) was swollen in the  
23 minimum volume of DMF until just freely mobile and  
24 dimethylamino pyridine (a few crystals) added. The  
25 activated peptide solution (2) was added and the  
26 coupling reaction sonicated for 4h. The mixture  
27 was then filtered and the resin washed with DMF,  
28 DCM and diethyl ether successively. The resin was  
29 dried under vacuum to give a final yield of 1.0g.  
30 The Fmoc loading test was carried out on the resin  
31 and a final loading of 0.162mmol/g was determined.  
32 It was established using Izumiya test that the

1 loading of the tripeptide onto the Wang resin was  
2 racemisation free.

3

#### 4 Synthesis of gp eotaxin on Wang resin

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6 The synthesis of gp eotaxin was carried out using  
7 500mg, 0.081mmol of resin (3). Standard coupling  
8 cycles using 1mmol of amino acid (HOt 2ml, 1mmol)  
9 and DIC (2ml, 1mmol) were carried out on the ABI  
10 synthesiser with the exception that:

11 a) the next amino acid Fmoc-Thr(Trt)-OH was  
12 coupled without a prior capping step on  
13 the resin and

14 b) the N-terminal amino acid Fmoc-His(Trt)-  
15 OH was coupled using HOBT 2mmol in place  
16 of HOt.

17

18 The final Fmoc group was retained on the resin as a  
19 purification tag.

20

#### 21 Cleavage, purification and isolation of gp eotaxin

22

23 After chain assembly, the Fmoc-peptide was cleaved  
24 with EDT/H<sub>2</sub>O/TIS/thioanisole/ TFA  
25 (0.5/1.0/0.2/0.2/10ml) at 0°C under nitrogen for  
26 4h. The resin was removed by filtration and peptide  
27 precipitated into cold ether and centrifuged. It  
28 was purified by G50 Sephadex gel filtration and  
29 HPLC and the amino terminal Fmoc group cleaved from  
30 the protein using 20% piperidine in CH<sub>3</sub>CN/H<sub>2</sub>O  
31 (1:1). DTT was added to reduce the side chain of  
32 Cys residues and the cleaved Fmoc removed by gel

1 filtration to give the pure, reduced peptide. This  
2 was folded in 50mM Tris pH8.0, 5mM GSH/0.5mM GSSG,  
3 and monitored by HPLC. Folding took about a week  
4 to complete.

5

6 The folded peptide was purified by HPLC, to give  
7 the pure, folded peptide. (Electrospray mass  
8 spectrometry; Expected mass 8356.9 Da, found 8353.9  
9 Da).

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